Attempt to Establish Field Colony of *Culex restuans* to Assess the Urban Heat Island Effect

Research Thesis

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By

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Abstract

The Urban Heat Island (UHI) effect explains why cities are typically warmer compared to surrounding suburban and rural areas. As mosquitoes are ectotherms, increases in temperatures can accelerate insect development, alter their phenology, and increase the disease incidence in urban settings. *Culex restuans* is a species of a mosquito commonly found in cities. Females of *Cx. restuans* transmit West Nile virus and enter a hibernation-like state, referred to as diapause, in response to short daylengths and low temperatures. Upon entering diapause, females cease reproductive development, stop blood feeding, and instead increase lipid accumulation, allowing them to survive without access to food during winter. Previous experiments have found that increases in temperature associated with the UHI effect during autumn inhibits diapause in a closely related species, *Cx. pipiens*. In this experiment, we hypothesized that high temperatures might also interfere with diapause initiation in *Cx. restuans.* To test this hypothesis, we attempted to establish a laboratory population of *Cx. restuans*. Oviposition traps were used to collect *Culex* egg rafts (F0) at three locations in central, Ohio, and larvae from the collected egg rafts were identified to species using morphology and PCR (polymerase chain reaction) assays. We then attempted to generate *Cx. restuans* F1 larvae to determine if temperatures that simulated the UHI effect in temperate biomes during winter months (20°C, 22°C, and 24°C) would inhibit diapause initiation. However, *Cx. restuans* that had emerged from field-collected rafts failed to successfully reproduce regardless of the presence of correct environmental cues and three different blood sources. Although we were unable to assess the impact of the UHI effect on diapause initiation in *Cx. restuans*, we found a high proportion of *Cx. restuans* within one urban site and a lower proportion within a rural site, as well as a high proportion of *Cx. pipiens* within a rural site. Difficulty establishing lab colonies of *Cx. restuans* limits our ability to study their

physiology. Considering the importance of *Cx. restuans* for local disease transmission and their notable prevalence in urban settings, especially during early summer, further work is needed to develop successful techniques to establish and rear this medically important insect pest.

1. Introduction

Regardless of significant efforts nationally to enact control methods, transmission of mosquito-borne illnesses remains an unfortunate problem. In the northeastern United States two mosquito species, *Culex restuans* and *Culex pipiens*, transmit several pathogens that cause serious diseases including West Nile virus (WNV), St. Louis encephalitis, Eastern equine encephalitis, and canine heartworm (Farajollahi et al. 2011). Each female *Culex* mosquito lays all of her eggs from a single feeding event in a connected egg raft that floats on the surface of water (Vinogradova 2000). Additionally, female *Culex* mosquitoes typically mate only once (Bullini et al. 1976), so the eggs within a raft are full siblings; thus, identification of only a handful of larvae from each raft is necessary to confirm species. Female mosquitoes enter a hibernation-like state called diapause in response to short daylengths and decreases in temperature (Sanberg and Larsen 1973). Once in diapause, disease transmission halts due to seasonal changes in *Culex* physiology and behavior (Eldridge 1987), with resulting effects including a shift away from blood feeding and reproduction and one towards survival.

Cities commonly exhibit high levels of impervious surfaces that radiate heat, generally causing higher temperatures compared to rural counterparts. This phenomenon is known as the Urban Heat Island (UHI) effect (Imhoff et al. 2009). The UHI effect is variable in degree of intensity, typically spanning from a 1-2 °C temperature difference between rural areas and urban cores but with a potential to differ up to 5-10°C depending on season, time of day, and the

surrounding biome (Imhoff et al., 2010; Pickett et al. 2001). As mosquitoes are ectotherms, temperature significantly impacts their physiology and lifespan. This is of public health interest as *Cx. pipiens* are typically more abundant in urban environments (Andreadis 2012), while *Cx. resutans* are generally colonize cleaner water in woodland pools and hence are common in rural areas (Crans 2014). Cities experience higher frequency of transmission of vector borne illnesses, including those transmitted by mosquitoes such as *Cx. pipiens* (Araujo et al. 2014, Ladeau et al. 2015). Commonly, *Cx. pipiens* are considered more anthropophilic than *Cx. restuans* who generally only bite avian species (Crans 2014), and therefore, West Nile virus risk to humans would be higher in areas where there are more *Cx. pipiens.* UHIs may influence the ability of *Culex* species to transmit diseases (Araujo 2014) as increased temperatures reduce incubation periods for West Nile virus and can additionally increase viral titers within *Cx. pipiens* (Vogels 2016, Dohm 2002, Kilpatrick 2008).

UHIs might also alter seasonal phenology in mosquitoes, especially as UHIs alter plant and animal physiology and phenology For example, the UHI effect causes numerous insect groups to reproduce and reach peak abundance earlier in the year due to advanced springtime phenology (Chick et al., 2019; Diamond et al., 2014; Hajdasz et al., 2019; Townroe and Callaghan, 2014; Villalobos-Jiménez and Hassall, 2017). Additionally, previous studies in our lab demonstrate that the UHI effect may also alter late-season phenology, and particularly diapause initiation in mosquitoes (Fyie et al. *in prep*). Specifically, we reared female *Cx. pipiens* under short-day conditions with temperatures that were 2°C and 4°C higher than diapause-inducing control conditions. The 4°C increase mimicked the average UHI effect present in the Midwest during the winter season as determined by Imhoff et al., (2009). We found that UHI-exposed adult female mosquitoes showed abnormal seasonal phenotypes with larger average egg

follicle sizes and increased proclivity to blood-feed. However, exposure to UHI-temperatures did not affect lipid accumulation.

In this study, we attempted to determine whether increased temperatures associated with UHIs would also affect diapause initiation in *Cx. restuans*. Since *Cx. restuans* are considered a more rural species, we suspected that they would be more sensitive to changes in temperature compared to *Cx. pipiens*. Therefore, we hypothesized that a lower percentage of *Cx. restuans* exposed to high temperatures would enter diapause, such that these females would have. large egg follicles and low levels of lipid.

2. Materials and Methods

Egg raft collection

Culex egg rafts were collected through oviposition traps at three locations throughout central Ohio including a rural orchard in Lancaster (Flowers of the Good Earth, 39.74317822270406, -82.61342881591388), an urban park in Columbus (Glen Echo Park, 40.0193140, -82.9993910), and an urban residental apartment complex in Columbus (107 E. 14th Avenue, 39.99859713934372, -83.0047582889214). Each egg raft was kept individualy in a clear, plastic container with distilled water inside an environmental chamber set to 27 °C and 14 hours of light and 10 hours of darkness. After larvae hatched and were a few days old, they were fed ground Tetramin fish food and identified to species using larval morphology and Polymerase Chain Reaction (PCR) assays.

Morphological identification of Culex larvae

Common *Culex* species within Ohio are Culex *pipiens*, *Culex restuans*, and *Culex salinarius*. Morphological identification was completed 2-4 days post hatching to allow for larval development so that we could easily visualize diagnostic characteristics: antennal tuft position, antenna coloration, and siphon length. Notably, *Cx. restuans* larvae are distinguished by central antennal hair tufts and a contrasting coloration of the antenna with a darker distal half and lighter proximal half (Figure 1a). The siphon of *Cx. restuans* larvae are relatively short in size (Fig. 1b). In contrast, *Cx pipiens* larvae are distinguished by their antennal hair tufts in the upper 1/3 region and a more uniform and even coloration of the antenna (Fig 2a), but have siphons that are comparable in length to *Cx. restuans* (Fig 2b). *Cx. salinarius* larvae (Fig. 3) are primarily distinguished by a significantly longer and more slender siphon (Fig 3b) compared to that of *Culex restuans* and *Culex pipiens*.

Figure 1

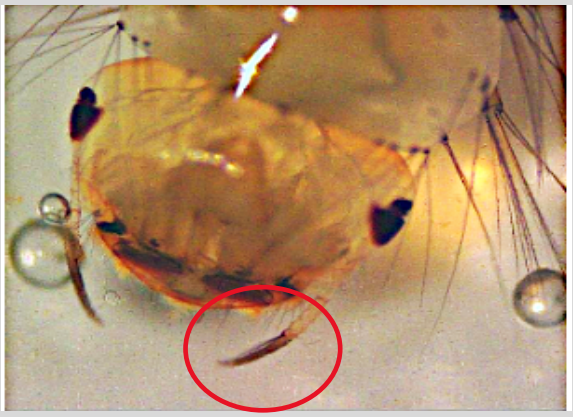


Figure 1a: *Culex restuans* larval head. The antenna is circled, showing the position of tufts and the contrast in color from the proximal to distal end *(Photo credit: H. Tronetti)*

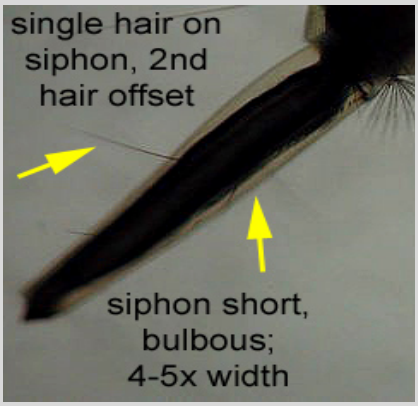


Figure 1b: *Culex restuans* larval siphon. Arrows indicate the position of hairs and the short and wide siphon. *(Photo credit: Florida Medical Entomology Department)*

Figure 2

Figure 2a: *Culex pipiens* larval head. The antenna is circled, showing the position of tufts and the uniformity in color from the proximal to distal end *(Photo credit: Florida Medical Entomology Department)*

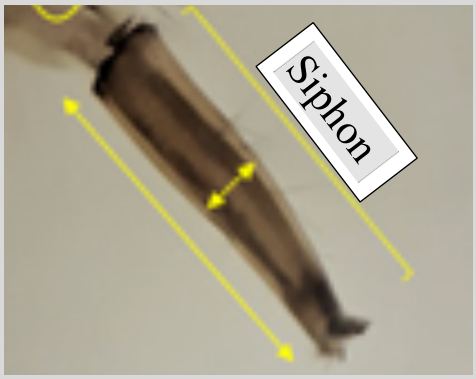


Figure 2b: *Culex pipiens* larval siphon. Arrows indicate the position of hairs and the short and wide siphon. *(Photo credit: Florida Medical Entomology Department)*

Figure 3

Figure 3a: *Culex salinarius* larval head. Head region depicted for comparison. *(Photo credit: Florida Medical Entomology Department)*

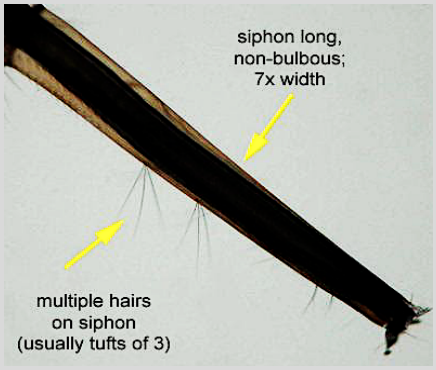


Figure 3b: *Culex salinarius* larval siphon. Arrows indicate the long and narrow siphon. *(Photo credit: Florida Medical Entomology Department)*

Polymerase Chain Reaction

A Polymerase Chain Reaction (PCR) assay was then completed in a subset of 10 larvae from 10 random egg rafts to validate the morphological identification. Genomic DNA was extracted from the whole individual at roughly 2 weeks after larvae hatched and was isolated using the Phire Tissue Direct PCR (Thermo Scientific) according to the manufacturer's protocol. PCR reactions were set up using 19 µl of DreamTaq Supermix (Fisher Scientific), 500 nm of Culex universal forward and *dd* specific Rev primers (Crabtree et al.1995), and 1 ul of gDNA (20 uL total reaction). Species-specific primers to distinguish *Cx restuans* are based on variation in nucleic acid sequence between the first and second internal transcribed spacers (ITS1 and ITS2) of the nuclear ribosomal protein 6 (Crabtree et al. 1995). This assay produced bands of 506 base pairs in *Cx. restuans* (Fig. 4). If *Cx. pipiens* genomic DNA had been present, a band at 698 bp would have been amplified. Fragments were amplified in a T100 thermocycler (BioRad) for 40 cycles with each cycle containing the following conditions: 98°C for 5 seconds, 60°C for 5 seconds, and 72°C for 20 seconds. Fragments were separated on a 1% agarose gel made with 1X Borax buffer by applying 200 V for approximately 45 minutes.

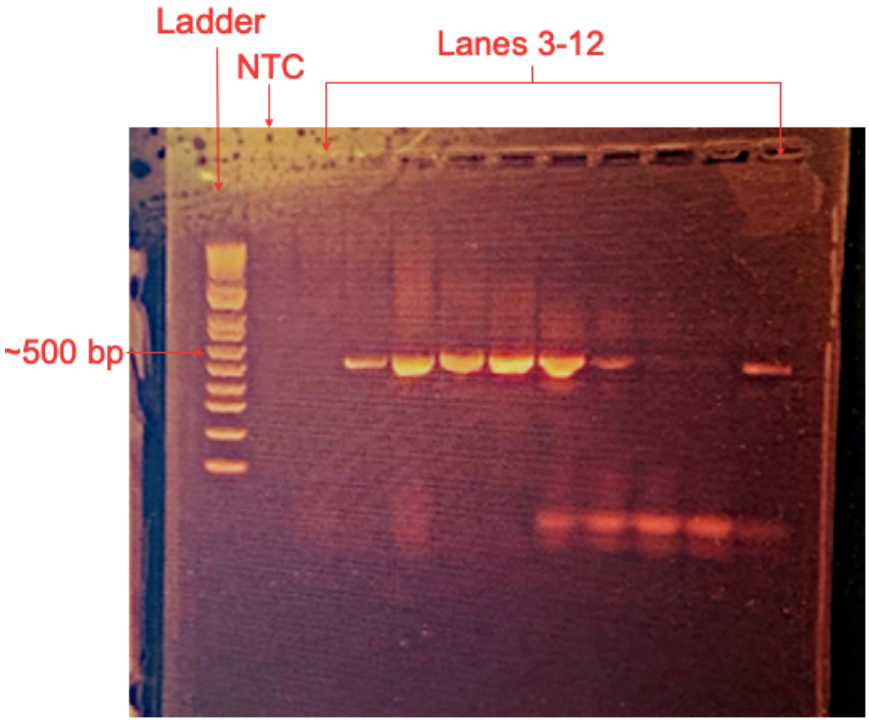


Figure 4: Representative gel after PCR amplification of *Culex restuans*-specific ribosomal DNA. Lanes 4-9 and lane 12 demonstrate amplification at ~506 bp indicating a positive Cx. restuans species identification. Lanes 8-12 demonstrate an additional amplified band further down the gel, most likely due to primer dimer formation due to insufficient sample DNA. The NTC or no template control served as a negative control reaction without DNA to check for reagent contamination. (*Photo credit: H Tronetti*)

Blood feeding Culex restuans

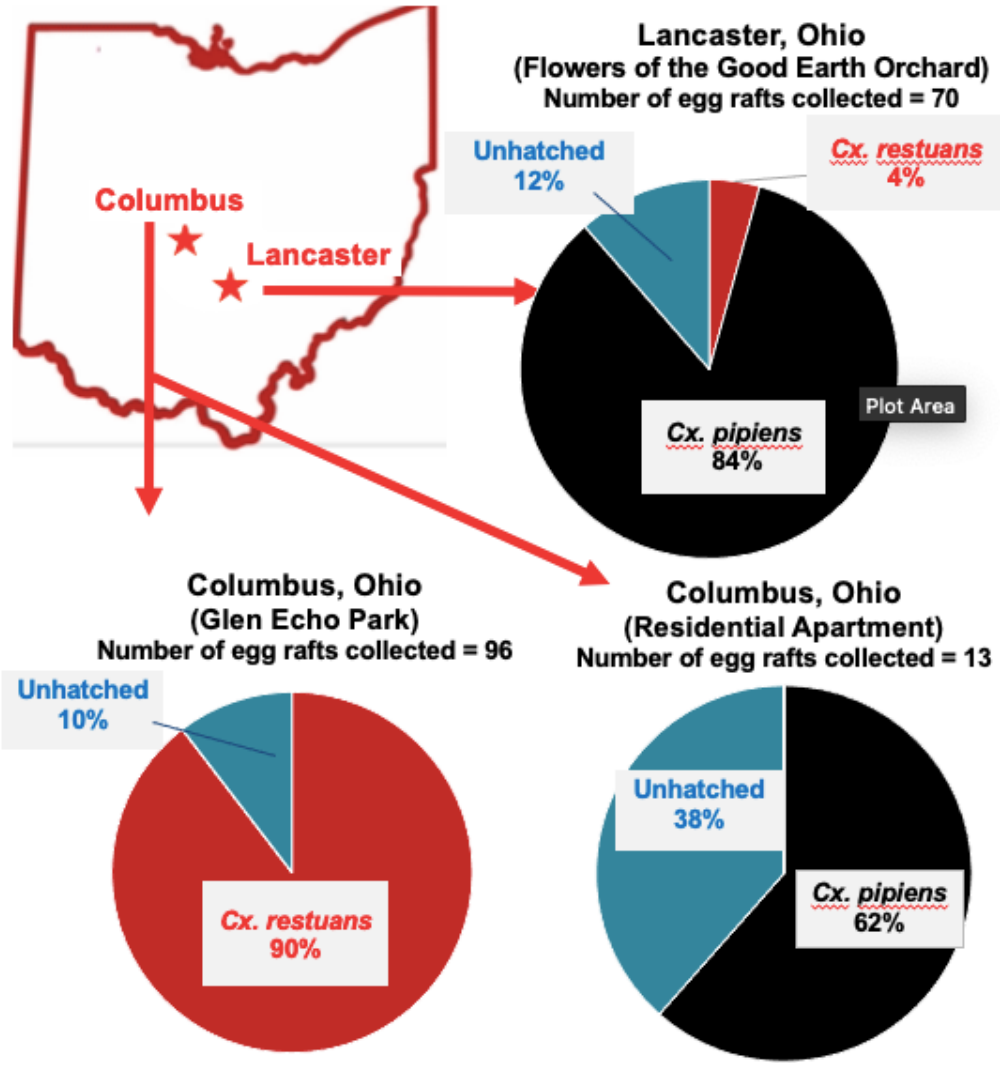
Once confirmed through morphological identification and PCR, larvae of *Cx. restuans* were combined in larger containers (8 oz). Pupae of *Cx. restuans* were picked and placed into mesh cages (11.25" x 11.25" x 9" Bioquip) where they emerged as adults. After emergence, adults were combined in a large plexi-glass cage (12 in. X 12 in. X 21.75 in.), and distilled water and sugar sources including 10% sucrose, honey, and raisins were provided *ad libitum*. To allow mosquitoes to mate before blood feeding, we waited until at least 7 days after peak adult emergence. We removed all sugar sources 24 hours before each round of blood feeding to

increase the likelihood that females would bite. At about 2 ½ - 3 weeks of age, adults were offered chicken blood spiked with 0.1M ATP in a Hemotek artificial membrane feeding system for 24 hours. A negligible percentage of females took a blood meal, and none produced egg rafts. Three days later, a human foot was offered for about 1 hour. Again, no females successfully blood fed. Five days after this, when the adults were close to 4 weeks of age, the females were offered a live chicken although again, no females took a blood meal. At this point, the females were over a month old, though one last attempt to blood feed was made through a different human foot which demonstrated no change in results. In the case of successful blood feeding and reproduction of the F0 population, F1 larvae would have been reared in environmental chambers set to a diapause-inducing photoperiod (11.5 hours of light and 12.5 hours of darkness) and temperatures of either 20°C (control), 22°C, or 24°C to simulate the UHI in temperature biomes during winter months (Imhoff citation; Fyie et al. *in prep*). The diapause status of F1 females would have been assessed by analyzing ovarian development and fat content between treatments, though the F0 population of *Culex restuans* failed to blood feed.

3. Results

Morphological identification proved very successful in terms of reliability as confirmed through PCR assay results. Unfortunately, the field-collected F0 population of *Cx. restuans* failed to reproduce and therefore we were unable to analyze the influence of the UHI effect on diapause initiation. However, data was collected regarding the distribution of *Culex* species throughout the month of June in central Ohio. Of the 96 egg rafts collected from Columbus's Glen Echo Park 86 successfully hatched and produced larvae, and all larvae were *Cx. restuans*. In contrast, 62 of the 70 egg rafts collected from the Flowers of the Good Earth, a rural orchard in Lancaster, Ohio,

successfully hatched and produced larvae, and 95% of these egg rafts (59) were identified as *Cx. pipiens* and the remaining 5% were *Cx. restuans*. Finally, of 13 collected egg rafts from an urban residential complex in Columbus, 8 egg rafts produced larvae which were all identified to be *Cx. pipiens*.



4.

Figure 5: Distribution of *Culex* species throughout various locations in Ohio during the month of June. Two urban locations in Columbus, Glen Echo Park and a residential complex, produced exclusively *Cx. restuans* or *Cx. pipiens* larvae. In contrast, in rural Lancaster, both *Cx. pipiens* and *Cx. restuans* were collected, although the majority of mosquitoes collected were *Cx. pipiens*.

Discussion

Morphological identification of *Culex* mosquito species based on characteristics of antennal coloration/hair tuft position and siphon length was observed to be reliable through confirmation of species by a PCR assay. Additionally, distribution of mosquitoes in rural and urban regions of Ohio including Lancaster and Columbus demonstrate high amounts of both *Cx. pipiens* and *Cx. restuans* during early summer months. *Cx. pipiens* have been regarded as more abundant in urban environments compared to suburban and rural areas (Andreadis 2012). However, our results demonstrated 84% of hatched larvae collected from egg rafts in a rural setting of Lancaster to be *Cx. pipiens*. On the other hand, in Glen Echo Park, which is located within Columbus city limits, 100% of the hatched collected egg rafts produced *Cx. restuans* larvae which was likewise not anticipated. Notably, the hatched egg rafts from the residential complex in Columbus were all *Cx. pipiens*, which is more consistent with the published literature. Although, it was interesting to observe the significant difference in *Culex* species prevalence between these two locations in Columbus which are approximately 2 miles apart. Overall, our data suggest that *Cx. pipiens* appear to be more common in urban and rural areas whereas *Cx. restuans* appear to be more common in urban parks, but it is shown through this experiment that their true distribution is more variable.

5. Future Directions

The difficulty establishing lab colonies of *Cx. restuans* limits our ability to study their physiology and thereby their ability to transmit disease. Considering their distinguished prevalence in urban settings, especially during early summer, further research is needed to acquire techniques to successfully establish and rear *Cx. restuans*. Potential changes could

include the implementation of an immobilized blood source to account for the increased sensitivity to movement wild mosquitoes often demonstrate. Additionally, the use of alternative avian blood, such as through using a house robin rather than a chicken, may provide better results. If these alterations fail, this experiment could be replicated through collected egg rafts from the field, placing them in individual cups and separating them out into incubators set to the various temperature treatments. Morphological and PCR analyses could then be conducted when they are old enough, and observations on egg follicle development and lipid accumulation could be collected.

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